DTIC FILE COPY

AD-A201 308

AD_____

DEVELOPMENT OF A SYSTEM OF STUDY GENE ACTIVATION IN MAMMALIAN CELLS TREATED WITH BIS (2-CHLOROETHYL) SULFIDE

Annual Report

Michael Lieberman, M.D., Ph.D.

December 1, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6066

Department of Pathology
Fox Chase Cancer Center
Philadelphia, PA 19111

DTIC
ELECTE
OCT 2 0 1988
S D
H

DOD DISTRIBUTION STATEMENT

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

88 10 20 013

SECURITY CLASSIFICATION OF THIS PAGE

ADA201308

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3 DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Fox Chase Cancer Center Department of Pathology; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Philadelphia, PA 19111	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-86-C-6066
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M16 2734A875; TASK NO. AA; WORK UNIT ACCESSION NO. 498

11. TITLE (Include Security Classification)
Development of a System to Study Gene Activation in Mammalian Cells Treated with Bis (2-Chloroethyl) Sulfide

12. PERSONAL AUTHOR(S)
Michael Lieberman, M.D., Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 11/1/86 TO 10/31/87	1987 December 1	17

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	01	
06	11	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
RA V; Cultured Mouse Cells; Gene Activation; Sulfur Mustard; Alkylating Agents; Metallothionein; Nucleic Acid Hybridization; Gene Rearrangement; DNA Methylation

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

It is known that treatment of mouse S49 thmic lymphoma cells with N-acetoxy-2-acetlamino-fluorene (NA-AAF) or ethylnitrosourea (ENU) activate two quiescent genes, metallothionein I (MT-I) and metallothnionein II (MT-II) (Annual Summary Report, 12/1/86). We reported that treatment with NA-AAF and ENU resulted in a 10-30 fold increase in cadmium-resistant variants and that about 65% of these variants made either MT-I, MT-II or both. Among other findings, we reported that the DNA methylation patterns (as measured by Hpa II digestion and Southern blotting) changed in lines which were MT positive. Using restriction endonuclease digestion and Southern blotting, we have not detected any insertions, deletions, rearrangements, or amplifications associated with the activation of either MT gene. → Z (1473 p. 2)

We have done initial experiments with sulfur mustard (SM) and established a range of toxicity for S49 cells. Sulfur mustard is extremely active in this system and produces as much as a 150-fold increase in the frequency of cadmium-resistant (Cd^r) variants compared to untreated control cells. We have rescued 22 variants from sulfur mustard treated populations and 9

Continued...

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia Miller	301/663-7325	SGRD-RMI-S

19. Continued

spontaneous variants and have made DNA and RNA from these. We are now in the process of analyzing metallothionein expression in these Cd^r variants.

DTIC
COPY
INSPECTED
4

Accession For
NTIS GRA&I ☑
DTIC TAB ☐
Unannounced ☐
Justification
By
Distribution/
Availability Codes
Dist | Avail and/or Special
A-1

SUMMARY

It is known that treatment of mouse S49 thymic lymphoma cells with N-acetoxy-2-acetylaminofluorene (NA-AAF) or ethylnitrosourea (ENU) activate two quiescent genes, metallothionein I (MT-I) and metallothionein II (MT-II) (Annual Summary Report, 12/1/86). We reported that treatment with NA-AAF and ENU resulted in a 10-30 fold increase in cadmium-resistant variants and that about 65% of these variants made either MT-I, MT-II or both. Among other findings, we reported that the DNA methylation patterns (as measured by Hpa II digestion and Southern blotting) changed in lines which were MT positive. Using restriction endonuclease digestion and Southern blotting, we have not detected any insertions, deletions, rearrangements, or amplifications associated with the activation of either MT gene.

We have done initial experiments with sulfur mustard (SM) and established a range of toxicity for S49 cells. sulfur mustard is extremely active in this system and produces as much as a 150-fold increase in the frequency of cadmium-resistant (Cd^r) variants compared to untreated control cells. We have rescued 22 variants from sulfur mustard treated populations and 9 spontaneous variants and have made DNA and RNA from these. We are now in the process of analyzing metallothionein expression in these Cd^r variants.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

TABLE OF CONTENTS

INTRODUCTION

As summarized in last year's annual summary report (1), both monofunctional and bifunctional alkylating agents can have profound effects on biological systems, including toxicity (cell death), mutation, and tumor induction. Attention has been focused on toxic and mutagenic aspects of these agents, but few investigators have examined the ability of these agents to activate unexpressed (quiescent) genes. We have shown previously (1,2) that ultraviolet radiation, NA-AAF, and ENU all can activate quiescent metallothionein genes in S49 thymic lymphoma cells. While the mechanism is unknown, it is clear that it involves demethylation of DNA and often coactivation of these two closely linked genes (3). In the present report we summarize data extending these results and analyzing the metallothionein gene region in NA-AAF and ENU Cd^r variants which make MT-I and/or MT-II. We also present initial data on SM which demonstrate that treatment of S49 mouse thymic lymphoma cells results in large increases in Cd^r variants.

MATERIALS AND METHODS

Cell culture. General cell culture procedures were as outlined in last year's report (1). Briefly we grew the cells in suspension in Ham's F12 medium supplemented with 10% fetal calf serum. 0.5% bottom agar (Difco) and 0.5% top low melt agarose (Bethesda Research Labs) were used with or without 5 μM Cd were used for selection for Cd^r variants and plating efficiency experiments. For sulfur mustard experiments SM diluted in absolute ethanol was added at the indicated final concentrations (see figures and tables) by diluting a small amount of absolute ethanol containing SM (approximately 10-25 μl) in 30 ml of medium containing approximately 1×10^6 S49 cells/ml. Procedures were done in screw cap 50 ml plastic centrifuge tubes in a chemical fume hood. After 3 hours incubation at 37°, the cells were spun down and resuspended in fresh medium.

Cells were either plated in soft agarose to determine relative survival (200-1000 cells/dish) or maintained in liquid medium for 5 days at 37° and 5% CO_2 to allow recovery. At this point cells were plated in soft agarose containing 5 μM $CdSO_4$ (10^6 cells/100 mm petri dish) or in soft agarose without cadmium for plating efficiency (200-1000 cells/dish). At the end of 14 days colonies were counted. Individual Cd^r colonies were rescued by aspiration into a sterile Pasteur pipette and seeding in liquid medium containing 5 μM $CdSO_4$. These were expanded and stocks were frozen down for future studies.

Nucleic Acid Procedures. DNA and RNA were isolated from guanidinium isothiocyanate/cesium chloride gradients (4). Restriction endonucleases were purchased from standard suppliers (e.g., Bethesda Research Laboratories, Amersham, New England Biolabs) and used as suggested by the manufacturer. Southern blots were performed in the standard fashion using ^{32}P nick-translated probes (4). Probes were made by isolating fragments from pBR322 plasmids containing pieces of the MT-I or MT-II genome (3,5).

RESULTS

Analysis of the Metallothionein Gene Region in MT^+ Variants. To determine if activation of the metallothionein genes was related to changes in genome structure, we analyzed 31 variants which were either $MT\text{-}I^+$, $MT\text{-}II^+$, or $MT\text{-}I^+/MT\text{-}II^+$. Variants were generated by treatment with NA-AAF or ENU and represent the same cell lines used in other experiments (1). We used three probes which span the metallothionein locus (Figure 1) and in separate experiments digested these variant lines with EcoRI, XbaI, PstI or BamHI. By a combination of these restriction endonucleases and probes we were able to generate eight different fragments which spanned approximately 25 kb of the mouse metallothionein locus from approximately 10 kb 5' of the MT-II gene (the 5' gene) to approximately 2 kb 3' of the MT-I gene (Table 1). When all of these variants were compared with

parental (untreated, cadmium-sensitive) S49 cells, we found no new bands and no changes in the size of existing bands. This finding indicates that there were no detectable deletions, insertions, or rearrangements using this method. There were no changes in the intensity of any of these bands. This finding and the failure to find changes in length indicates that there has been no significant amplification of any of the genes at this locus. In some instances (e.g., the 0.3 kb band expected from an EcoRI digestion probed with N1500) the method is quite sensitive, and we could have seen a change as small as 50 or 100 base pairs; in other instances the method is less sensitive (e.g. the 10 kb band expected from an XbaI digestion, probed with PP300), and changes much smaller than 1 kb might not have been visible. Nevertheless the data are fairly consistent and suggest that major changes in the primary gene sequence do not occur during activation of the metallothionein genes by these two monofunctional alkylating agents. However, small deletions or insertions or point mutations would not be detectable by this assay.

Toxicity of sulfur Mustard for S49 Cells. We performed several experiments to analyze the toxicity of sulfur mustard for S49 thymic lymphoma cells. We found that dilution of sulfur mustard in ethanol and the addition of small amounts (~10-25 μl) to large amounts of tissue culture medium (30 ml) gave reasonably reproducible results. The relative survival for one of these experiments is presented in Table 2 and graphed in Figure 2. A concentration of ~1 μM sulfur mustard results in about a 5% survival while ~0.5 μM and ~0.25 μM result in ~20% and 60% survival, respectively. It appears that there is a small shoulder in the log-log plot which suggests that there may be some repair of sulfur mustard damage in these cells.

Generation of Cd^r Colonies After sulfur Mustard Treatment. We allowed these cells to recover from SM treatment for 5 days in liquid medium and then plated

them in either soft agarose without cadmium for relative plating efficiency calculations or soft agarose containing 5 μM cadmium for cadmium resistance measurements (Table 3 and Figure 3). It is clear from these studies that the frequency of Cd^r variants in untreated S49 cells is ~$2.6/10^6$ cells while in survivors of the highest treatment (~1 μM) there is an ~140 fold increase. Thus sulfur mustard is very active in this system.

Preparation of Cells and Nucleic Acids from sulfur Mustard Induced Cd^r Variants. We have frozen down 18 variants derived from ~0.5 μM treated cells, 4 variants derived from ~1 μM treated cells and 9 lines (spontaneous variants) from untreated plates. We chose to focus most of our efforts on the ~0.5 μM generated variants since the 10-fold increase seen in going from ~0.5 μM SM to ~1 μM SM raises the possibility of more than one hit per locus. While potentially interesting, we chose to focus our attention on the less complicated case. Since at ~0.5 μM SM we see a 14 fold increase in variants over spontaneous levels, it is reasonable to assume that most of the variants we have isolated result from treatment with SM at this dose level. We have made DNA and RNA from all of these and are in the process of running northern blots and Southern blots to anzlyze these for MT-I and MT-II expression as well as DNA methylation and alterations in the metallothionein gene region.

DISCUSSION

It is clear from our previous data (1,2) and our present data (Results section) that ultraviolet radiation and two monofunctional alkylating agents result in large increases in Cd^r colonies in S49 cells, many of which make MT-I, MT-II, or both. Changes in DNA methylation are associated with activation of the MT genes, but no simple pattern of methylation change can be directly related to the activation of these genes (1). An extension of this analysis (Figure 1, Table 1) in which we looked for insertions, deletions, rearrangements, or amplifications associated with the activation of these genes, failed

to yield any changes. It is possible that activation is the result of point mutations or small deletions or insertions which the present methodology cannot detect. In theory it is possible to use probes designed to detect mismatches to analyze these events (6,7). This method has been used successfully to detect ras mutations in nucleic acids from patients with colon cancers. However, unlike that study in which only about 300 base pairs had to be examined, in the present study there is no indication where such mutations might exist. Thus, at the present time, it seems inappropriate to apply this methodology. Another way in which these monofunctional alkylating agents might activate the MT genes is by inducing changes in chromatin structure which are passed from one generation to the next. It is well known that during development the activation of genes is accompanied by changes in chromatin structure as well as DNA methylation. Although it is possible to analyze changes in chromatin structure (e.g., analysis of DNase I or staphylococcal nuclease hypersensitive sites), at present there are no well developed strategies for looking at the mechanisms of these changes. Thus although it is clear that all of the above agents can activate these two quiescent genes, the analysis of the mechanism of activation will prove difficult.

Although working out of the logistics of obtaining SM in the proper vehicle took more time than expected, we have made good progress in analyzing the effects of SM in the S49/metallothionein system. We found that SM is very active in this system. The toxicity of SM for S49 cells is somewhat great than that observed for Hela cells (8). Following treatment with SM, there is a large increase in Cd^r variants. At present we are in the process of characterizing these variants, and it will be interesting to determine how many are MT-I^+, MT-II^+, MT-I^+/MT-II^+, and MT$^-$. We already have good data on the distribution of variants in these classes for cells treated with ENU and NA-AAF, and the comparison with SM variants will be useful. Likewise, analysis of DNA methylation and genomic structural changes should be helpful in determining if SM

produces a different spectrum of DNA changes. Most important, however, is the observation itself: treatment with SM results in the conversion of cells from cadmium-sensitive to cadmium-resistant and thus probably activates quiescent metallothionein genes. If substantiated by further analysis, our findings will provide the basis for a new approach to the analysis of the biological effects of sulfur mustard.

REFERENCES

1. Lieberman, M.W. (1986) Annual Summary Report Contract Number DAMD17-86-C-6066.

2. Lieberman, M.W., Beach, L.R., and Palmiter, R.D. (1983) Ultraviolet radiation-induced metallothionein-I gene activation is associated with extensive DNA demethylation. Cell 35, 207-214.

3. Searle, P.F., Davison, B.L., Stewart, G.W., Wilkie, T.M., Norstedt, G. and Palmiter, R.D. (1984) Regulation, linkage and sequence of mouse metallothionein I and II genes. Mol. Cell. Biol. 4, 1221-1230.

4. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Isolation of total cellular RNA. In Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

5. Durnam, D.M., Perrin, F., Gannon, F., and Palmiter, R.D. (1980) Isolation and characterization of the mouse metallothionein-I gene. Proc. Natl. Acad. Sci. USA 77, 6511-6515.

6. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M., van Boom, J.H., van der Eb, A.J., and Vogelstein, B. (1987) Prevalence of ras gene mutations in human colorectal cancers. Nature 327, 293-297.

7. Forrester, K., Almoguera, C., Han, K., Grizzle, W.E., and Perucho, M. (1987) Detection of high incidence of K-ras oncogenes during human colon tumorigenesis. Nature 327, 298-303.

8. Craethorn, A.R., and Roberts, J.J. (1966) Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. Nature 211, 150-153.

Table 1: Fragment length from Southern Blot Analysis of Parental (MT^-) and Variant (MT^+) S49 Cells

Gene	Expected Band* in kb	Enzyme**	Probe**
MT-I	3.8	EcoRI	KB600
MT-I	10.0	XbaI	PP300
MT-II	9.0, 0.7, 0.3	EcoRI	N1500
MT-II	4.0, 2.1	PstI	N1500
MT-I/MT-II	15.0	BamHI	KB600

*In all cases the expected and observed fragment lengths were the same.

**See Figure 1 for map.

Table 2: Survival of S49 Cells Following Sulfur Mustard Treatment*

Final SM Concentration	Soft Agarose Colonies/400 Plated Cells	Cd^r colonies/ Relative Survival**
0	373,383	1.0
0.26 μM	219,229	0.59
0.53 μM	100,116	0.28
1.06 μM	14,26	0.05

*See Materials and Methods and Legend to Figure 2 for details.

**Computed by averaging the values in column 2 and dividing by the value for 0 treatment.

Table 3: Appearance of Cd^r Following Sulfur Mustard Treatment*

Final SM Concentration	Relative Plating Efficiency	Cd^r colonies/ 10^6 cells plated**	Cd^r colonies/ 10^6 Survivors***
0	1.0	2.6	2.6
0.26 μM	1.10	6.9	11.7
0.53 μM	1.14	10.5	37.5
1.06 μM	1.02	18.5	370.0

*See Materials and Methods and legend to Table 2 for details.

**Corrected for plating efficiency.

***Corrected for relative survival (Table 2).

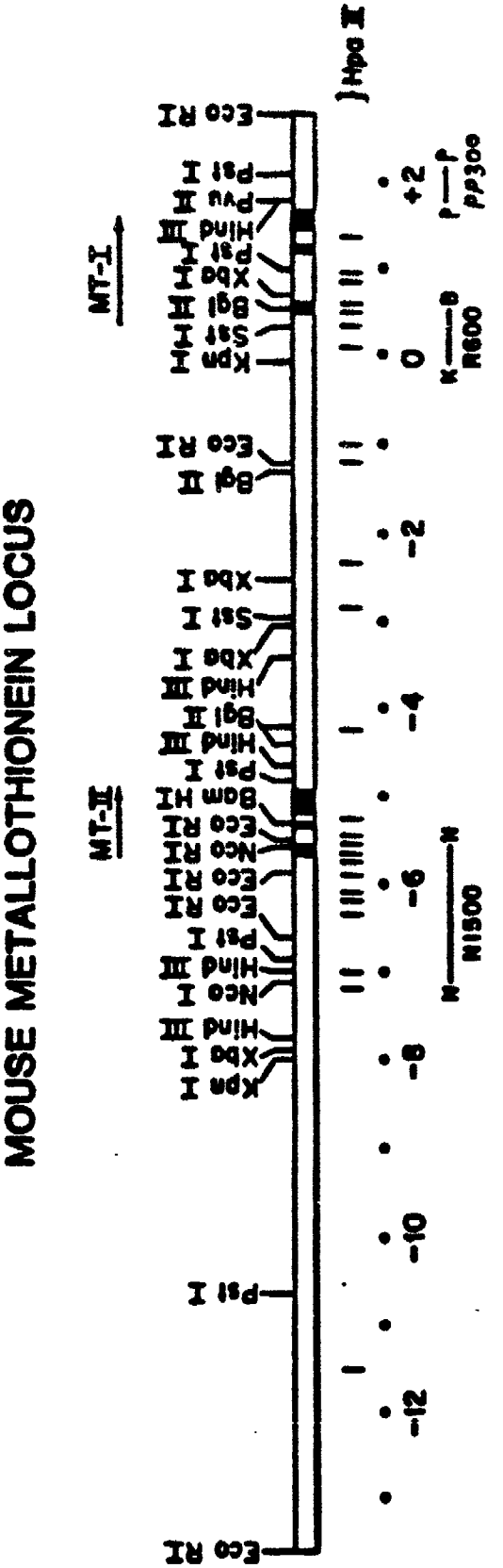
MOUSE METALLOTHIONEIN LOCUS
MT-I
MT-II
Eco RI
Pst I
Kpn I
Xba I
Hind III
Nco I
Eco RI
Eco RI
Nco I
Bam HI
Pst I
Hind III
Bgl II
Xba I
Sst I
Xba I
Bgl II
Eco RI
Kpn I
Sst I
Bgl II
Xba I
Hind III
Pst I
Pvu II
Eco RI
Hpa I
-12
-10
-8
-6
-4
-2
0
+2
N1500
R600
pp300

Figure 1

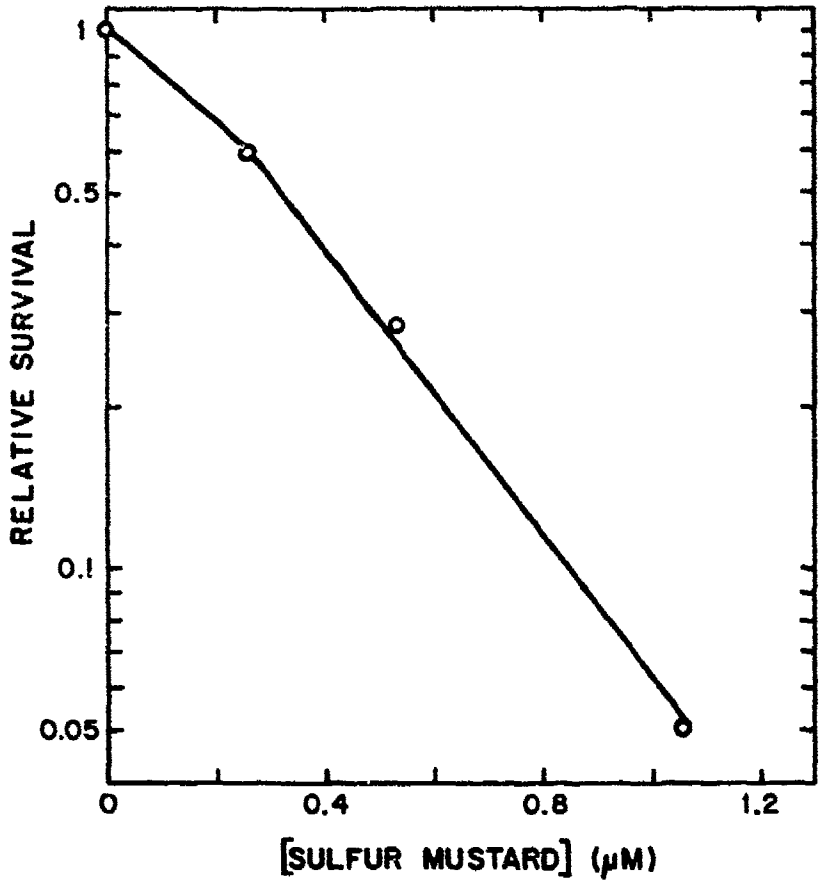

Figure 2. Toxicity of sulfur mustard for S49 mouse lymphoma cells. S49 cells were treated in Ham's F12 medium with 10% fetal calf serum at the indicated molarities of sulfur mustard for three hours at 37°. Following treatment, cells were plated at 400 cells per 100 mm dish in 0.5% soft agarose. Colonies were counted at 14 days.

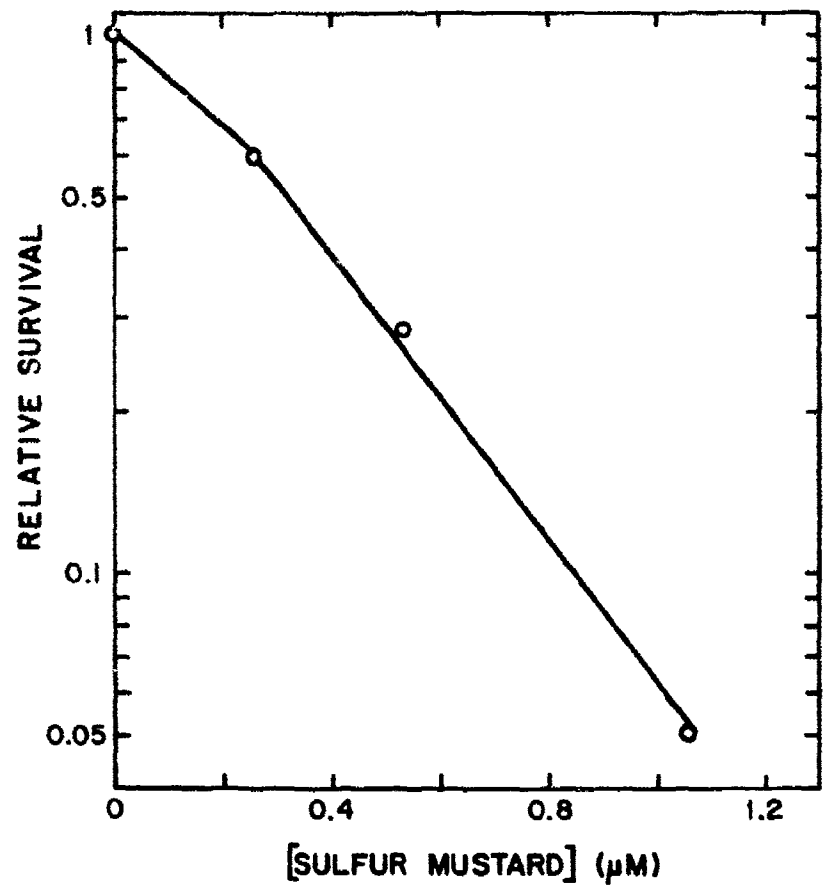

Figure 2. Toxicity of sulfur mustard for S49 mouse lymphoma cells. S49 cells were treated in Ham's F12 medium with 10% fetal calf serum at the indicated molarities of sulfur mustard for three hours at 37°. Following treatment, cells were plated at 400 cells per 100 mm dish in 0.5% soft agarose. Colonies were counted at 14 days.

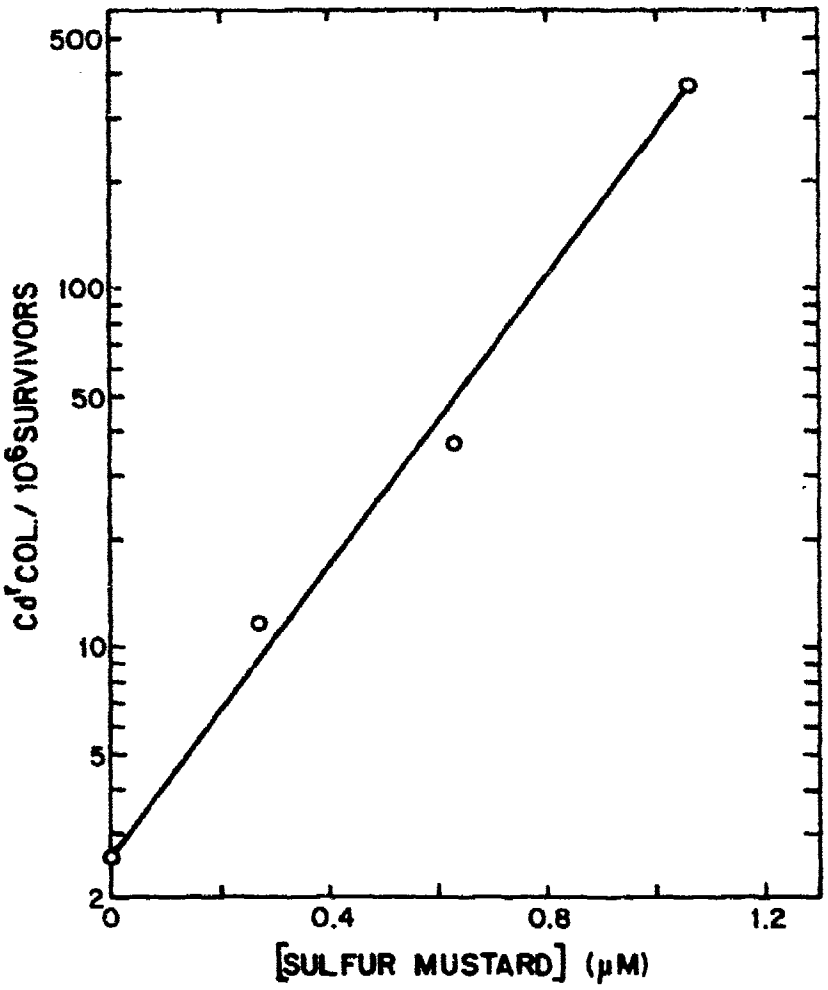

Figure 3. Appearance of Cd^r following sulfur mustard treatment. Cells were treated with sulfur mustard as described in the text and legend to Figure 2 and allowed to recover for 5 days in liquid medium. They were then plated at 10^6 cells per 100 mm petri dish in 0.5% soft agarose containing 5 μM Cd (5 dishes per dose). Similar aliquots for plating efficiency were plated at 200 to 1,000 cells per dish in agarose without Cd. The number of Cd^r colonies in each dish was counted at 14 days and corrected for relative survival (Figure 2).